(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 382 018 A1**

(12) **EUROPEAN PATENT APPLICATION**
published in accordance with Art. 153(4) EPC

(43) Date of publication:
03.10.2018 Bulletin 2018/40

(21) Application number: **16868667.3**

(22) Date of filing: **25.11.2016**

(51) Int Cl.:
C12N 15/09 *(2006.01)* ***C07K 16/18*** *(2006.01)*
C12N 1/15 *(2006.01)* ***C12N 1/19*** *(2006.01)*
C12N 1/21 *(2006.01)* ***C12N 5/10*** *(2006.01)*
C12N 9/10 *(2006.01)*

(86) International application number:
PCT/JP2016/084958

(87) International publication number:
WO 2017/090724 (01.06.2017 Gazette 2017/22)

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR
Designated Extension States:
BA ME
Designated Validation States:
MA MD

(30) Priority: **25.11.2015 JP 2015229896**

(71) Applicant: **National University Corporation Gunma University**
Maebashi-shi, Gunma 371-8510 (JP)

(72) Inventors:
- **HATADA, Izuho**
  Maebashi-shi
  Gunma 371-8510 (JP)
- **MORITA, Sumiyo**
  Maebashi-shi
  Gunma 371-8510 (JP)
- **HORII, Takuro**
  Maebashi-shi
  Gunma 371-8510 (JP)

(74) Representative: **Gill Jennings & Every LLP**
The Broadgate Tower
20 Primrose Street
London EC2A 2ES (GB)

(54) DNA METHYLATION EDITING KIT AND DNA METHYLATION EDITING METHOD

(57) A DNA methylation editing kit comprises: (1) a fusion protein of inactivated CRISPR-associated endonuclease Cas9 (dCas9) having no nuclease activity and a tag peptide array in which plural tag peptides are linked by linkers, or an RNA or DNA coding therefor; (2) a fusion protein(s) of a tag peptide-binding portion and a methylase or demethylase, or an RNA(s) or DNA(s) coding therefor; and (3) a guide RNA(s) (gRNA(s)) comprising a sequence complementary to a DNA sequence within 1 kb of a desired site of methylation or demethylation, or a DNA(s) expressing the gRNA(s).

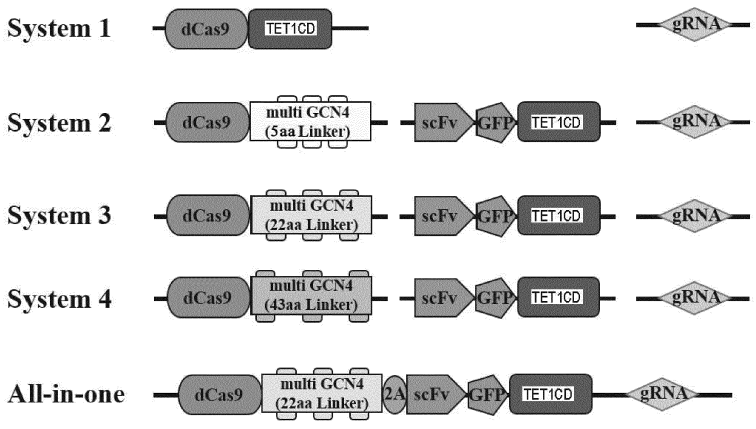

FIG.1

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Description

Technical Field

[0001] The present invention relates to a DNA methylation editing kit and a DNA methylation editing method.

Background Art

[0002] The methylation of cytosine in genomic DNA is a typical modification of epigenetics (epigenome) regulating gene expression. Possible regulation of the methylation of a particular gene enables elucidation of epigenome diseases such as cancer, production of models of the diseases, and its application to epigenome treatment. Currently, treatment of cancer using the demethylation of the whole genome with 5-azacytosine or the like is put into practical use. However, the treatment affects all genes, and therefore, some doubt remains as to safety concerns. Therefore, development of a technology for regulating the methylation of a particular site has been desired.

[0003] As such a technology for regulating the methylation of a particular site, a technology for demethylating a particular gene by using a protein obtained by fusing TALEN and the catalytic domain of TET1 which is an enzyme involved in demethylation has been previously reported (Non Patent Literature 1). However, it was very time-consuming due to use of TALEN, which is a genome editing technology of the previous-generation, and the degree of demethylation has not been very high.

[0004] Examples of new-generation genome editing methods include a method of using CRISPR/Cas (Non Patent Literature 2). Although use and application of an array in which plural peptide epitopes are linked, and scFv which is a single-chain antibody for signal amplification have been reported (Non Patent Literature 3) as a CRISPR/Cas genome editing method, the method has not been known to be applied to regulation of DNA methylation.

Citation List

Non Patent Literature

[0005]

Non Patent Literature 1: Maeder ML et al. Nat Biotechnol, 31, 1137-1142, 2013
Non Patent Literature 2: JIKKEN IGAKU (YODOSHA CO., LTD.), July, 2014, pp. 1690-1714
Non Patent Literature 3: Tanenbaum ME et al. Cell 159, 635-646, 2014

Summary of Invention

Technical Problem

[0006] In view of the problems described above, an object of the present invention is to provide a DNA methylation editing kit and a DNA methylation editing method.

Solution to Problem

[0007] As a result of intensive study for solving the problems described above, the present inventors found that use of a CRISPR/Cas genome editing method enables the methylation of a particular site to be effectively regulated, and the present invention was thus accomplished.

[0008] In other words, the gist of the present invention is as follows.

[1] A DNA methylation editing kit comprising:

(1) a fusion protein of inactivated CRISPR-associated endonuclease Cas9 (dCas9) having no nuclease activity and a tag peptide array in which a plurality of tag peptides are linked by linkers, or an RNA or DNA coding therefor;
(2) a fusion protein(s) of a tag peptide-binding portion and a methylase or demethylase, or an RNA(s) or DNA(s) coding therefor; and
(3) a guide RNA(s) (gRNA(s)) comprising a sequence complementary to a DNA sequence within 1 kb of a desired site of methylation or demethylation, or a DNA(s) expressing the gRNA(s).

[2] The DNA methylation editing kit according to [1], wherein the demethylase is a catalytic domain (TET1CD) of

ten-eleven translocation 1.

[3] The DNA methylation editing kit according to [1], wherein the methylase is DNA methyltransferase 3 beta (DNMT3B).

[4] The DNA methylation editing kit according to any one of [1] to [3], wherein the tag peptides are peptide epitopes, and the tag peptide-binding portion is an anti-peptide-epitope antibody.

[5] The DNA methylation editing kit according to [4], wherein the peptide epitopes are general control non-derepressible 4 (GCN4) peptide epitopes, and the anti-peptide-epitope antibody is an anti-GCN4 peptide epitope antibody.

[6] The DNA methylation editing kit according to [4], wherein the peptide epitopes are His tags or EE tags, and the anti-peptide-epitope antibody is an anti-His tag antibody or an anti-EE tag antibody.

[7] The DNA methylation editing kit according to any one of [4] to [6], wherein the antibody is a single-chain antibody (scFv).

[8] The DNA methylation editing kit according to any one of [1] to [3], wherein the tag peptides are a small fragment of a split protein, and the tag peptide-binding portion is a large fragment of the split protein.

[9] The DNA methylation editing kit according to [8], wherein the split protein is GFP.

[10] The DNA methylation editing kit according to any one of [1] to [3], wherein the tag peptides are GVKESLV, and the tag peptide-binding portion is PDZ protein.

[11] The DNA methylation editing kit according to any one of [1] to [10], wherein the linkers consist of 5 to 100 amino acids.

[12] The DNA methylation editing kit according to any one of [1] to [11], wherein the linkers consist of 5 to 50 amino acids.

[13] The DNA methylation editing kit according to any one of [1] to [12], wherein the linkers consist of 10 to 50 amino acids.

[14] The DNA methylation editing kit according to any one of [1] to [13], wherein the fusion proteins of the (1) and/or (2) further include a selection marker.

[15] The DNA methylation editing kit according to any one of [1] to [14], which contains plural gRNAs

[16] The DNA methylation editing kit according to any one of [1] to [15], wherein all the DNAs of the (1) to (3) are contained in one vector.

[17] A DNA methylation editing method comprising transfecting a cell with the following (1) to (3):

(1) a fusion protein of inactivated CRISPR-associated endonuclease Cas9 (dCas9) having no nuclease activity and a tag peptide array in which a plurality of tag peptides are linked by linkers, or an RNA or DNA coding therefor;
(2) a fusion protein(s) of a tag peptide-binding portion and a methylase or demethylase, or an RNA(s) or DNA(s) coding therefor; and
(3) a guide RNA(s) (gRNA(s)) comprising a sequence complementary to a DNA sequence within 1 kb of a desired site of methylation or demethylation, or a DNA(s) expressing the gRNA(s).

[18] The DNA methylation editing method according to [17], wherein the fusion proteins of the (1) and/or (2) further include a selection marker.

[19] The DNA methylation editing method according to [18], further comprising selecting and collecting a cell expressing the selection marker.

Advantageous Effects of Invention

[0009] According to the present invention, it is possible to regulate the DNA methylation of a particular site, for example, to demethylate a methylated site, and to methylate an unmethylated site.

Brief Description of Drawings

[0010]

[Figure 1] Figure 1 is a view illustrating the components of transfected vectors (Example 1).

[Figure 2] Section (a) of Figure 2 is a view illustrating a STAT3 binding site and a mouse Gfap site. The STAT3 binding site has a methylation-sensitive CpG site (CG in TTCCGAGAA)). Targets 1 to 3 used as gRNAs (Gfap1-3) are indicated by black thick bars. Section (b) of Figure 2 is a graph illustrating the demethylation activity of dCas9 (system 1) directly bound to a TET1 catalytic domain (TET1CD) in which gRNAs targeting Gfap1-3 are used. The ordinate represents a value calculated by the Numerical Formula in the table (the same as the Numerical Formula 1 shown below) as a standardized demethylation percentage (%).

[Figure 3A] Figure 3A is a view illustrating a scheme of demethylation amplification based on dCas9 and a repeating

peptide array. Inactivated Cas9 (dCas9) fused with the repeating peptide array and having no nuclease activity can recruit plural pieces of scFv antibody-fused TET1CD. Therefore, the plural pieces of TET1CD can more effectively demethylate a target.

[Figure 3B] Figure 3B is a view illustrating a case in which the length of a linker separating each GCN4 peptide epitope fused with dCas9 is too short (left), a case in which the length is appropriate (center), and a case in which the length is too long (right).

[Figure 4] In section (a) of Figure 4, the ordinate represents a value calculated by the Numerical Formula 1 shown below as a standardized demethylation percentage (%). The abscissa represents the system of a vector used and the presence or absence of sorting. Target 2 of Gfap was used as a gRNA. Section (b) of Figure 4 is a view illustrating the methylation in the peripheries of target sites. ESCs transfected with gRNAs targeting system 3 and Gfap2 or a control gRNA were sorted by GFP, and methylation was analyzed by bisulfite sequencing. A black-and-white-style circle represents the percentage of the methylation, and the black represents methylation while the white represents unmethylation. The number under the circle represents each position. Statistical significances between all CpG site sets in the two groups (Gfap and control) were evaluated by Mann-Whitney U test.

[Figure 5] Figure 5 is a view illustrating the methylation in the peripheries of off-target sites 1 to 3 of a gRNA targeting Gfap2. ESCs transfected with gRNAs targeting system 3 and Gfap2 were sorted by GFP, and the methylation of the peripheries of the off-target sites 1 to 3 was analyzed by bisulfite sequencing. A black-and-white-style circle represents the percentage of the methylation, and the black represents methylation while the white represents unmethylation. The number under the circle represents each position. Statistical significances between all CpG site sets in the two groups (Gfap and control) were evaluated by Mann-Whitney U test. The underlined portions of the sequences represent portions in which Gfap2 targets and nucleotide sequences match with each other.

[Figure 6] Section (a) of Figure 6 illustrates CTCF binding sites and a mouse H19 site. The CTCF binding sites have methylation-sensitive CpG sites (m1 to m4). Sites 1 to 4 used in the targets of gRNAs are illustrated under vertically long bars representing m1 to m4. Section (b) of Figure 6 illustrates the demethylation of m2 in the CTCF binding sites using systems 1 and 3, and system 3 + sorting. The ordinate represents a value calculated by the Numerical Formula 1 shown below as a standardized demethylation percentage (%). The abscissa represents the system of a vector used and the presence or absence of sorting. Section (c) of Figure 6 is a graph illustrating the demethylation in the CTCF binding sites (m1 to m4) in the case of using system 3 + sorting. Left and right bars in each site of m1 to m4 represent demethylation in the case of using the target site 2 as a gRNA and demethylation in the case of using all the gRNAs of the target sites 1 to 4 together, respectively. The ordinate represents a value calculated by the Numerical Formula 1 shown below as a standardized demethylation percentage (%).

[Figure 7] Figure 7 is a view illustrating the components of transfected vectors (Example 2).

[Figure 8] Section (a) of Figure 8 illustrates CTCF binding sites and a mouse H19 site. The CTCF binding sites have methylation-sensitive CpG sites (m1 to m4). In Example 2, m2 was used as a target. Section (b) of Figure 8 illustrates the methylation of m2 in the CTCF binding sites using system 3 + sorting. The ordinate represents a value calculated by the following Numerical Formula 2 as a standardized methylation percentage (%).

Description of Embodiments

[0011] Embodiments of the present invention will be described in detail below.

[0012] In CRISPR/Cas, Cas9, which is a DNA-cleaving enzyme, forms a complex with a short RNA (guide RNA (gRNA)) comprising an about-20-bp sequence complementary to a target, and cleaves DNA as a target (Non Patent Literature 2). In such a case, when a mutant enzyme having no DNA cleavage activity, referred to as dCas9, is used, only binding to a target can be achieved without cleaving the target. Thus, recruitment of factors that perform methylation and demethylation by linking various components to dCas9 enables the methylation of a particular gene to be regulated. When a system where dCas9 linked with a tag peptide array comprising plural tag peptides, and a tag peptide-binding portion such as a single-chain antibody (scFv) for a tag peptide fused with a factor performing methylation and demethylation are used, and plural methylation factors or demethylation factors can be recruited for one molecule of dCas9, and an ability to perform the methylation or demethylation can be enhanced (Figure 3a).

[0013] In the present invention, first, a sequence (target sequence) complementary to a DNA sequence within 1 kb from a desired site of methylation or demethylation is produced, and a gRNA comprising the target sequence is produced. The gRNA has a property of forming a complex, with dCas9 having no nuclease activity.

When a fusion protein of dCas9 and a tag peptide array is produced, the gRNA forms a complex with the fusion protein, through dCas9, and therefore, a gRNA-dCas9- tag peptide array complex is formed. The gRNA is bound to a sequence complementary to a target sequence included in the gRNA, and therefore, the gRNA-dCas9- tag peptide array complex is bound to a DNA sequence within 1 kb from a desired site of methylation or demethylation. A fusion protein of a tag peptide-binding portion and a methylase or demethylase is recruited within 1 kb from the desired site of methylation or demethylation by binding of the tag peptide-binding portion to the tag peptide array. The recruited methylase or demeth-

ylase methylates or demethylates a site within 1 kb from its recruited portion (Figure 3a).

(DNA Methylation Editing Kit and DNA Methylation Editing Method)

[0014] The present invention relates to a DNA methylation editing kit comprising: (1) a fusion protein of inactivated CRISPR-associated endonuclease Cas9 (dCas9) having no nuclease activity and a tag peptide array in which plural tag peptides such as GCN4 are linked by linkers, or an RNA or DNA coding therefor; (2) a fusion protein(s) of a tag peptide-binding portion such as an anti-tag peptide antibody and a methylase or demethylase, or an RNA(s) or DNA(s) coding therefor; and (3) a guide RNA(s) (gRNA(s)) comprising a sequence complementary to a DNA sequence within 1 kb from a desired site of demethylation, or a DNA(s) expressing the gRNA(s). In addition, the present invention relates to a DNA methylation editing method comprising transfecting a cell with the (1) to (3) described above.

[0015] The DNA methylation editing includes both of the methylation of a DNA unmethylated site and the demethylation of a DNA methylated site.

(Inactivated Cas9 Having No Nuclease Activity)

[0016] CRISPR-associated endonuclease Cas9 (Cas9) includes two lobes of a REC lobe (REC: recognition) and a NUC lobe (NUC: nuclease), in which the NUC lobe is a site responsible for nuclease activity (Non Patent Literature 2). Thus, inactivated Cas9 (dCas9) having no nuclease activity in the present invention can be produced by introducing a mutation into the NUC lobe of Cas9. As a result, the nuclease activity of Cas9 can be inactivated while maintaining the capacity of binding to a target site. A site in which the mutation is introduced into the NUC lobe is not limited as long as only the nuclease activity can be inactivated. For example, mutation of Asp10 to alanine (D10A), mutation of His840 to alanine (H840A), and mutation of Asn863 to alanine (N863A) in Cas9 (UniProtKB/Swiss-Prot: Q99ZW2) are preferred. Such mutations may be one kind or a combination of two or more kinds thereof.

[0017] DNAs encoding dCas9 can be produced by introducing mutations into DNAs encoding Cas9 that can be obtained from GenBank and the like. Alternatively, plasmids comprising commercially available dCas9 may be obtained from Addgene and the like and used, DNAs encoding dCas9 may be obtained by PCR with the plasmids as templates or may be artificially produced using an artificial gene synthesis technology known to those skilled in the art, and methods of obtaining the DNAs are not limited. RNAs encoding dCas9 may be obtained by known molecular biological techniques, of which any may be used. For example, such an RNA may be obtained by using a DNA encoding the dCas9 as a template and triggering an RNA polymerase.

(Tag Peptide Array)

[0018] The tag peptide array in the present invention refers to a tag peptide array in which plural tag peptides are linked by linkers.

[0019] The tag peptides can be optionally selected in combination with a tag peptide-binding portion described later. Examples of the combination of the tag peptides and the tag peptide-binding portion include a combination of a peptide epitope and an antibody recognizing the peptide epitope, and a combination of the small fragment and large fragment of a split protein.

[0020] Examples of the combination of a peptide epitope and an antibody recognizing the peptide epitope include: GCN4 and an anti-GCN4 antibody; a His tag and an anti-His tag antibody; an EE hexapeptide and an anti-EE hexapeptide antibody; a c-Myc tag and an anti-c-Myc tag antibody; an HA tag and an anti-HA tag antibody; an S tag and an anti-S tag antibody; and a FLAG tag and an anti-FLAG tag antibody (Protein Engineering, Design & Selection vol. 24 no. 5 pp. 419-428, 2011). Among them, a peptide included in GCN4 is preferably used, the amino acid sequence of GCN4 can be obtained from, for example, PDB, and the DNA sequence of GCN4 can be obtained from GenBank or the like. Those skilled in the art can also obtain an RNA sequence corresponding to the DNA sequence on the basis of information on the DNA sequence by using nucleotide sequence conversion software and the like. The GCN4 peptide epitope can be used without limitation as long as being an epitope in GCN4, and an amino acid sequence represented by SEQ ID NO: 1 is preferred. Information on the amino acid sequences of the other tag peptides and the nucleotide sequences encoding the amino acid sequences can be obtained from known databases and the like.

[0021] The split protein refers to a pair of proteins in which, in the case of dividing a certain protein into two portions, the two portions of the protein are reassociated, thereby enabling formation of the same structure as that of the original protein. Particularly in the case of dividing the original protein into the two portions, one portion as a short peptide (small fragment) may be used with a tag peptide, and the other longer portion (large fragment) may be used as a tag peptide-binding portion (Current Opinion in Chemical Biology 2011, 15: 789-797). A known split protein can be used as the split protein which can be used for such a purpose, and examples thereof include GFP (green fluorescent protein).

[0022] Further, binding of a peptide and a protein domain is compiled into a database, and a combination of a tag

peptide and a tag peptide-binding portion can be found with reference to, for example, Peptide Binding Proteins Database (http://pepbind.bicpu.edu.in/home.php). For example, since PDZAlpha-Syntrophin PDZ protein interaction domain can be bound to GVKESLV (SEQ ID NO: 44), GVKESLV can be used with a tag peptide, and the PDZ domain can be used as a tag peptide-binding portion.

[0023] Further, the binding strength of a pair of a peptide and a peptide binding portion can be increased by connecting another unrelated domain with a linker and performing domain interface evolution. Methylation can be further efficiently regulated by using such a pair (Proc. Natl. Acad. Sci. USA, 2008, vol. 105 no. 18, 6578-6583).

[0024] Linkers interposed in a tag peptide array comprising such plural tag peptides as described above include any sequence as long as the linkers do not inhibit binding of the peptides and peptide-binding portions or the desired effect of the present invention. Examples of the linkers include a repeating sequence of glycine and serine. The length of such a linker can be set as appropriate according to the kind of a methylase or demethylase and the like, and is preferably 5 to 100 amino acids, more preferably 5 to 50 amino acids, and still more preferably 10 to 50 amino acids. In the case of TET and DNMT described later, the length is more preferably 15 to 40 amino acids, still more preferably 17 to 30 amino acids, and most preferably 22 amino acids. When the length of the linker is 10 amino acids in the case of the repeating sequence of glycine (G) and serine (S), for example, the linker sequence may be GSGSG (SEQ ID NO: 45), GSGSGGSGSGSGGSGSGGSGSG (SEQ ID NO: 46), or GSGSGGSGSGGSGSGGSGSGGSGGSGSGGSGSGGSGSGGSGSG (SEQ ID NO: 47).

[0025] The tag peptide array in the present invention refers to a tag peptide array in which assuming that a combination of a tag peptide and a linker is one unit, one or plural units are repeatedly linked. The plural units mean two or more units. The number of repeated units can be increased or decreased as appropriate depending on the distance between a target site and a methylated or demethylated site, the kind of a methylase or demethylase, and the like, and may be, for example, 3 to 5.

[0026] DNA encoding a tag peptide array can be produced by adding a DNA sequence encoding a desired linker to DNA encoding a tag peptide that can be obtained from GenBank or the like. A method of obtaining the DNA by a molecular biological technique based on information on a DNA sequence is known. For example, the DNA can be artificially produced using an artificial gene synthesis technology known to those skilled in the art, and the method of obtaining the DNA is not limited. Those skilled in the art can also obtain an RNA sequence corresponding to the DNA sequence on the basis of information on the DNA sequence by using nucleotide sequence conversion software and the like.

(Fusion Protein of dCas9 and Tag Peptide Array, or RNA or DNA Coding Therefor)

[0027] DNA encoding a fusion protein of dCas9 and a tag peptide array can be produced by binding of DNA encoding the dCas9 defined above and DNA encoding the tag peptide array by using an optional method including a well-known gene manipulation method, and is not particularly limited. A DNA sequence encoding a selection marker may also be inserted into the DNA encoding the fusion protein. The selection marker enables cells into which the DNA encoding the fusion protein is introduced to be selected by cell sorting or the like. Examples of the selection marker include, but are not limited to, genes encoding fluorescent proteins such as GFP, Ds-Red, and mCherry, and drug resistance genes such as puromycin resistance genes and neomycin resistance genes. The fusion protein or RNA encoding the fusion protein can be obtained by a known molecular biological technique using DNA encoding the fusion protein, and can be obtained by, for example, inserting DNA encoding the fusion protein into an appropriate expression vector and expressing the protein or the RNA.

(Tag peptide-binding portion)

[0028] As the tag peptide-binding portion, an anti-tag peptide (peptide epitope) antibody, the large fragment of a split protein, or the like can be used depending on the kind of a tag peptide, as described above. The anti-tag peptide antibody means an antibody that specifically recognizes a tag peptide. The anti-tag peptide antibody includes polyclonal antibodies and monoclonal antibodies. The monoclonal antibodies include monoclonal antibodies, the fragments of monoclonal antibodies, $F(ab')_2$ antibodies, F(ab') antibodies, short-chain antibodies (scFv), diabodies, and minibodies. DNA encoding the anti-tag peptide antibody can be obtained by a known molecular biological technique, can be obtained by amplifying, for example, a commercially available plasmid such as Addgene plasmid 60904 by PCR, or may be artificially produced using an artificial gene synthesis technology known to those skilled in the art, and a method of obtaining the DNA is not limited. The anti-tag peptide antibody or RNA encoding the anti-tag peptide antibody can be obtained by inserting the DNA encoding the anti-tag peptide antibody into an appropriate expression vector and expressing the protein or the RNA.

(Methylase and Demethylase)

[0029] The methylase in the present invention can be used without limitation as long as being an enzyme that catalyzes

the methylation of an unmethylated site, and includes a methylase which is an enzyme that methylates a particular base on a DNA nucleotide sequence, and a methyltransferase which is an enzyme transferring a methyl group to a particular base, and more specific examples thereof include DNA methyltransferase 3 beta (DNMT3B), DNA methyltransferase 3 alpha (DNMT3A), and DNA methyltransferase 1 (DNMT1). The demethylase in the present invention can be used without limitation as long as being an enzyme catalyzing a series of reaction leading to the demethylation of a methylation site, and includes ten-eleven translocation 1 (TET1), ten-eleven translocation 2 (TET2), ten-eleven translocation 3 (TET3), and thymine-DNA glycosylase(TDG). These enzymes may be a portion or the whole of an enzyme protein. Preferred examples of the portion of the enzyme protein include a catalytic domain of an enzyme. Information on the sequence of DNAs encoding the enzymes can be obtained from GenBank and the like, and the DNAs can be produced from the cDNAs of target animals such as human by PCR. Alternatively, the DNAs encoding the enzymes may be artificially produced using an artificial gene synthesis technology known to those skilled in the art, and methods of obtaining the DNAs are not limited. The enzymes or RNAs encoding the enzymes can be obtained by inserting the DNAs into an appropriate expression vector and expressing the proteins or RNAs.

(Fusion Protein of Tag peptide-binding portion and Methylase or Demethylase, or RNA or DNA Encoding Fusion Protein)

[0030] DNA encoding a fusion protein of a tag peptide-binding portion such as an anti-peptide-epitope antibody and a methylase or demethylase can be produced by linking DNA encoding the tag peptide-binding portion defined above with DNA encoding a methylase or demethylase by using an optional method including a well-known gene manipulation method, and is not particularly limited. A DNA sequence encoding a selection marker may also be inserted into DNA encoding the fusion protein. The selection marker enables cells into which the DNA encoding the fusion protein is introduced to be selected by cell sorting or the like. Examples of the selection marker include, but are not limited to, genes encoding fluorescent proteins such as GFP, Ds-Red, and mCherry, and drug resistance genes such as puromycin resistance genes and neomycin resistance genes. When a DNA sequence encoding a selection marker is inserted into the DNA encoding the fusion protein of the dCas9 and the tag peptide array, a selection marker different from the selection marker may be inserted into DNA encoding a fusion protein of a tag peptide-binding portion and a methylase or demethylase. The fusion protein or RNA encoding the fusion protein can be obtained by a known molecular biological technique using DNA encoding the fusion protein, and can be obtained by, for example, inserting DNA encoding the fusion protein into an appropriate expression vector and expressing the protein or the RNA.

(Guide RNA (gRNA) or DNA Expressing Guide RNA)

[0031] The guide RNA (gRNA) in the present invention is a guide RNA in which a tracrRNA and a crRNA are artificially linked in a CRISPER/Cas method. By a known technique based on an RNA sequence described in Non Patent Literature 2 (p. 1698), DNA corresponding to the RNA sequence can be obtained as DNA expressing tracrRNA. For example, the DNA may be artificially produced using an artificial gene synthesis technology known to those skilled in the art, and a method of obtaining the DNA is not limited. Alternatively, a plasmid that enables a desired gRNA to be expressed by inserting a DNA sequence corresponding to an arbitrary crRNA is commercially available (Addgene plasmid 41824 or the like) and may be therefore used. A sequence complementary to a DNA sequence within 1 kb from a desired site of methylation or demethylation is used as the crRNA. One kind of the gRNA is acceptable, or plural gRNAs each comprising different crRNAs may be used.

(All-in-One Vector)

[0032] The DNAs encoding the two fusion proteins described above may be further linked, resulting in DNA encoding a fusion protein of dCas9, a tag peptide array, a tag peptide-binding portion, and a methylase or demethylase, which may be incorporated into a vector and may be used. The vector comprising the DNA is referred to as an all-in-one vector. A linker may be inserted as appropriate into the DNA encoding the fusion protein. For example, when a 2A peptide derived from a virus is inserted as a linker between a fusion protein (regarded as a component 1) of dCas9 and a tag peptide array and a fusion protein (regarded as a component 2) of a tag peptide-binding portion and a methylase or demethylase, the 2A peptide is cleaved by the 2A peptidase in a cell, and therefore, the components 1 and 2 are prevented from being linked and expressed as two separated proteins. The all-in-one vector may also include a gRNA.

[0033] Examples of vectors comprising desired genes in the present invention include a vector that can be replicated in a eukaryotic cell, a vector which maintaining an episome, and a vector incorporated into a host cell genome, and viral vectors are preferred, and adenovirus vectors, lentiviral vectors, and adeno-associated virus vectors are more preferred. Such a vector may include a selection marker. "Selection marker" refers to a genetic element which provides a selectable phenotype to a cell into which the selection marker is introduced, and is commonly a gene of which a gene product imparts resistance to an agent that inhibits cell proliferation or kills or wounds a cell. Specific examples thereof include

Neo gene, Hyg gene, hisD gene, Gpt gene, and Ble gene. Examples of a drug useful for selecting the presence of the selection marker include G418 for Neo, hygromycin for Hyg, histidinol for hisD, xanthine for Gpt, and bleomycin for Ble.

(Transfection into Cell)

[0034] Transfection of DNA, RNA, and a protein into a cell can be performed by using known optional means or may be performed using a commercially available reagent for transfection. For example, electroporation, Lipofectamine 2000 (Invitrogen), jetPRIME Kit (Polyplus-transfection), DreamFect (OZ Biosciences), GenePorter3000 (OZ Biosciences), Calcium Phosphate Transfection Kit (OZ Biosciences), and the like can be used for transfection of DNA. Electroporation, Lipofectamine 3000 (Invitrogen), RNAi Max (Invitrogen), MessengerMAX (Invitrogen), and the like can be used for transfection of RNA. Electroporation, Lipofectamine CRISPRMAX (Invitrogen), PULSin (Polyplus-transfection), Pro-DeliverIN (OZ Biosciences), BioPORTER Protein Delivery Reagent (Genlantis), and the like can be used for transfection of a protein. Transfection into a cell may also performed by forming a complex of a gRNA and a fusion protein of dCas9 and a tag peptide array, in advance, and transfecting the complex into the cell. DNA, RNA, or a protein can also be introduced into a fertilized egg by microinjection or electroporation.

EXAMPLES

[0035] The present invention will be further described below with reference to non-limiting examples. In the present examples, GCN4 was used as a tag peptide. However, the GCN4 can be replaced with another tag peptide.

Example 1. Demethylation of Target Using TET1CD

<Plasmid Construction for Target Demethylation>

[0036] A dCas9-TET1 catalytic domain (CD) fusion protein expression vector (pCAG-dCas9TET1CD) was produced by fusing cDNA encoding codon-optimized *S.pyogenes* Cas9 (dCas9) as a catalytically inactive nuclease to a catalytic domain in the N-terminus of human TET1CD (System 1). A dCas9 fragment was amplified from Addgene plasmid 48240 by PCR. A TET1CD fragment was amplified from human cDNA by PCR.

[0037] The dCas9 fragments of Systems 1 to 4 in Figure 1 were amplified from Addgene plasmid 60903 by PCR. The amino acid sequence of used GCN4 was EELLSKNYHLENEVARLKK (SEQ ID NO: 1). Linker sequences between GCN4 are GSGSG (SEQ ID NO: 2: System 2), GSGSGGSGSGSGGSGSGGSGSG (SEQ ID NO: 3: System 3), and GSGSGGSGSGGSGSGGSGSGGSGGSGSGGSGSGGSGSGGSGSG (SEQ ID NO: 4: System 4). A GFP fragment was amplified from Addgene plasmid 60904. An ScFv fragment was also amplified from Addgene plasmid 60904. All the fusion proteins were expressed under the control of a CAG promoter. An all-in-one vector was produced by fusing a 2A peptide (SEQ ID NO: 5: GSGATNFSLLKQAGDVEENPGP) into between the components 1 and 2 of System 3. Vector sequences are shown in SEQ ID NOS: 6 to 11 (which are System 1 (pCAG-dCas9TET1CD), dCas9-GCN4 fusion protein of System 2 (pCAG-dCas9-10xGCN4_v4), scFv-TET1CD fusion protein of Systems 2 to 4 (pCAG-scFvGCN4sfGFPTET1CD), dCas9-GCN4 fusion protein of System 3 (pCAG-dCas9-5xPlat2AflD), dCas9-GCN4 fusion protein of System 4 (pCAG-dCas9-3.5xSuper), and all-in-one vector (pPlatTET-gRNA2), respectively).

<Construction of gRNA>

[0038] A gRNA vector for Gfap or H19 was produced by inserting a target sequence into Addgene plasmid 41824. Cloning was performed by Gibson assembly system via the linearization of an AflII site and the insertion of a gRNA fragment.

[0039] Target sequences are set forth in Table 1.

Table 1: Target Sequence

Target Name	Target Sequence	Methylation-Sensitive Site around Target
Gfap_1	ATAGACATAATGGTCAGGGGTGG	Gfap STAT3-binding site
Gfap_2	GGATGCCAGGATGTCAGCCCCGG	Gfap STAT3-binding site
Gfap_3	ATATGGCAAGGGCAGCCCCGTGG	Gfap STAT3-binding site
H19DMR_1	GTGGGGGGGCTCTTTAGGTTTGG	H19DMR CTCF-binding site 1
H19DMR_2	ACCCTGGTCTTTACACACAAAGG	H19DMR CTCF-binding site 2

(continued)

Target Name	Target Sequence	Methylation-Sensitive Site around Target
H19DMR_3	GAAGCTGTTATGTGCAACAAGGG	H19DMR CTCF-binding site 3
H19DMR_4	CAGATTTGGCTATAGCTAAATGG	H19DMR CTCF-binding site 4

The underlines show PAM sequences.

Unrelated gRNA Sequence

Target Name	gRNA Sequence
UR_1	CCATTATTGCATTAATCTGA
UR_2	TAATGCAGCCAGAAAATGAC
UR_3	TCAGGGATCAAATTCTGAGC

<Cell Culture>

[0040] Embryonic stem cells (ESCs) were cultured in Dulbecco's modified Eagle's medium-high-concentration glucose (D6429-500ML, Sigma) to which 1% FBS, 17.5% KSR100 (10828028, Gibco), 0.2% of 2-mercaptoethanol (21985-023, Gibco), and 1×10^3 unit /mL (ESG1107, Millipore) of ESGRO mLIF were added under 37°C and 5% CO_2. The ESCs were transfected using Lipofectamine 2000 (Invitrogen) according to an attached protocol, and the cells were collected 48 hours after the transfection and directly used for an assay and a sort by FACSAriaII (BD Biosciences).

<DNA Methylation Analysis>

[0041] Genomic DNA was treated using Epitect Plus DNA Bisulfite Kit (QIAGEN) according to an attached instruction. The modified DNA was amplified using the following PCR primers in Table 2.

Table 2: PCR Primer Sequence for Bisulfite Sequence

Primer Name	Primer Sequence	Methylation-Sensitive Site around Target
GfapSTAT3-B3	TTGGTTAGTTTTTAGGATTTTTTTT	Gfap STAT3-binding site (ES)
GfapSTAT3-B4	AAAACTTCAAACCCATCTATCTCTTC	
H19DMR-B1	AAGGAGATTATGTTTTATTTTTGGA	H19DMR CTCF-binding site 1
H19DMR-B2	AAAAAAACTCAATCAATTACAATCC	
Gfap_O1B1	TTGTAAAGGTAGGATTAATAAGGGAATT	Gfap off-target site 1
Gfap_O1B2	AAAAAAAACCCTTCAAAAAAAATCTA	
Gfap_O2B1	TTATTATTTATATTTGGAGGGAGGG	Gfap off-target site 2
Gfap_O2B2	ATTACACCAAAAAAATTTTAAAAAC	
Gfap_O3B1	TTTAAATTTTTTTATGTGAATATGG	Gfap off-target site 3
Gfap_O3B2	AAACATTTAATTCATTAATACACAC	

[0042] The percentages of the demethylation of the STAT3 site of Gfap and the m1 to m4 sites of H19 were determined by Combined Bisulfite Restriction Analysis (COBRA). The fragments amplified using the primers in Table 3 were cleaved with restriction enzymes having recognition sites in the sites and set forth in in Table 3 below and subjected to polyacrylamide gel electrophoresis.

Table 3: COBRA Primer Sequence

primer name	primer sequence	Restriction enzyme	methylation sensitive site near the targets
GfapSTAT3-B1	GTTGAAGATTTGGTAGTGTTGAGTT	Hpy188III	Gfap STAT3-binding site
GfapSTAT3-B2	TAAAACATATAACAAAAACAACCCC		
H19DMR-B1	AAGGAGATTATGTTTTATTTTTGGA	BstUI	H19DMR CTCF-binding site 1
H19DMR-B2	AAAAAAACTCAATCAATTACAATCC		
H19DMR-B1	AAGGAGATTATGTTTTATTTTTGGA	RsaI	H19DMR CTCF-binding site 2
H19DMR-B2	AAAAAAACTCAATCAATTACAATCC		
H19DMR-B3	GGGTTTTTTTGGTTATTGAATTTTAA	BstUI	H19DMR CTCF-binding site 3
H19DMR-B4	AATACACACATCTTACCACCCCTATA		
H19DMR-B5	TTTTTGGGTAGTTTTTTTAGTTTTG	BstUI	H19DMR CTCF-binding site 4
H19DMR-B6	ACACAAATACCTAATCCCTTTATTAAAC		

[0043] The methylation was calculated as the ratio of cleaved DNA by densitometry analysis of a gel stained with ethidium bromide. In each assay, the methylation of cells transfected with a control vector (empty gRNA vector) was defined as 100% methylation (0% demethylation), and the demethylation of each sample was standardized by the control using the following Numerical Formula 1.

Numerical Formula 1

$$\text{Demethylation of sample (\%)} = (\text{methylation of control} - \text{methylation of sample})/\text{methylation of control} \times 100$$

[0044] Bisulfite sequencing was carried out for the methylation analysis and off-target analysis of a peripheral region. The amplified fragment was ligated into a TOPO vector (Invitrogen), and sequencing of at least 14 clones was carried out. The sequencing was analyzed by a methylation analysis tool referred to as QUantification tool for Methylation Analysis (QUMA). Statistical significance between two groups of all sets in CpG sites was evaluated using Mann-Whitney U test (also referred to as Wilcoxon matched pairs signed ranks test is called) used for a test of nonparametric statistical significance.

<Results>

[0045] First, a simple design which was a direct fusion protein of inactivated Cas9 nuclease (dCas9) and TET1 was produced for methylation treatment. TET1 has a catalytic domain preserved in a C-terminus, and this domain has higher catalytic activity than that of a full-length protein. Therefore, the TET1 catalytic domain (TET1CD) was fused to dCas9 having inactive catalytic action (System 1 in Figure 1).

[0046] A cytosine residue in a STAT3-binding site located upstream of a gene encoding glial fibrillary acidic protein (GFAP) which is an astrocyte-specific marker was used as a target. The site is methylated in many cell types excluding astrocytes, and the demethylation of the site plays an important role in differentiation of neural precursor cells (NPCs) into astrocytes. Three targets around the STAT3-binding site were designed (Figure 2a), and a gRNA vector for the targets was produced. The gRNA vector was transiently introduced, together with a dCAS9-TET1CD fusion protein expression vector (pCAG-dCas9TET1CD), into embryonic stem cells (ESCs). The methylation of the STAT3-binding site was analyzed by Combined Bisulfite Restriction Analysis (COBRA). In each assay, the methylation of cells into which a gene was introduced together with a control vector (empty gRNA vector) was defined as 0% demethylation (100% methylation), and the demethylation of each sample was standardized by the control.

[0047] In the STAT3 site, the three gRNAs, Gfap1, Gfap2 and Gfap3, showed demethylations of 3%, 14%, and 9%, respectively (Figure 2b). In contrast, the unrelated gRNAs (UR1, UR2, and UR3) showed no demethylation. Thus, this simple system induced gRNA-dependent specific demethylation, but the degree of the demethylation was shown to be at most 14%.

[0048] Then, an attempt to amplify a demethylation ability was made using dCas9 fused in a repeating peptide sequence in order to recruit plural copies of the antibody fused TET1 hydroxylase catalytic domain (Figure 3a). For the demethylation of the Gfap STAT3 site, an expression vector of Gfap2gRNA, dCas9 having 10 copies of GCN4 peptides, and a GCN4 peptide antibody (scFv)-superfolder green fluorescent protein (sfGFP)-TET1CD fusion protein was used in ESCs (System 2 in Figure 1). However, the use of this System 2 did not allow the degree of the demethylation to be improved (Figure 4a).

[0049] The length of a linker by which the sequence of a GCN4 peptide epitope comprising 19 amino acids was separated was examined in order to investigate the reason why System 2 failed to improve the degree of the demethylation. If the length of the linker is too short, it is considered that for the antibody-TET1CD fusion protein, a space for approaching and binding to the GCN4 peptide sequence is too narrow, and therefore, demethylation activity becomes insufficient. If the length of the linker is too long, it is considered that the antibody-TET1CD fusion protein is incapable of approaching a target methylated site (Figure 3b). The length of the linker of System 2 was 5 amino acids (System 2 in Figure 1).

[0050] An antibody-TET1CD fusion protein having a linker of which the length was 22 amino acids (System 3 in Figure 1) and a TET1CD fusion protein having a linker of which the length was 43 amino acids (System 4 in Figure 1) were produced, and the demethylation activities thereof were compared. Because of technological limitation in a synthetic gene technology, the numbers of copies of GCN4 peptides having a linker of which the length was 22 amino acids and a linker of which the length was 43 amino acids were decreased to 5 and 4, respectively. In spite of the decreases in the numbers of the copies of the GCN4 peptides, the linker of which the length was 22 amino acids showed a best demethylation of 43%. The linker of which the length was 44 amino acids showed a second highest activity, and the linker, as a prototype, of which the length was 5 amino acids showed the lowest activity (Figure 4a).

[0051] These results suggested that the length of a linker by which each GCN4 peptide unit sequence fused with dCas9 is separated is more important for demethylation activity than the number of copies of GCN4. The demethylation activity was prominently improved by increasing the length of the linker from 5 amino acids to 22 amino acids. This is considered to be because the 22 amino acids have a width enough for the antibody-TET1CD fusion protein to approach a peptide sequence. In contrast, the linker of which the length was 43 amino acids was considered to be long for the antibody-TET1CD fusion protein to approach a methylated site which was a target.

[0052] Cells into which a GFP expression vector was introduced were selected using fluorescence activated cell sorting (FACS) for the purpose of further improving demethylation efficiency. For this purpose, an all-in-one vector comprising a gRNA, dCas9 comprising the GCN4 sequence of System 3, and an antibody-sfGFP-TET1CD fusion protein was produced (Figure 1). The all-in-one introduced ESCs sorted by GFP showed roughly complete demethylation (Figure 4). The ESCs in to which System 3 was introduced and which was sorted by GFP also unexpectedly showed roughly complete demethylation (Figure 4). Complete demethylation in a target region was achieved by the promotion of the demethylation ability and the sorting technology.

[0053] Then, the range of the demethylation of a used sorted sample from a target site was investigated by bisulfite sequencing. The demethylation occurred even at a site located at least 100 bp or more apart from the target site (Figure 4b). Investigation of off-target activity by bisulfite sequencing using the same sample resulted in no observation of noticeable off-target activity (Figure 5).

[0054] Then, a similar experiment was conducted using a differential methylation region (DMR) of H19 as a paternal methylated imprinting gene. The DMR of H19 includes four methylation-sensitive CTCF binding sites (m1 to m4), which are important for adjusting H19 imprinting (Figure 6a). A gRNA (H19DMR2) targeting m2 was introduced, together with dCas9-TET1CD or System 3, into ESCs. The cells into which System 3 had been introduced and which were subjected to cell sorting after the introduction were also produced.

[0055] As a result, noticeable improvement in methylation in System 3 was observed in comparison with dCas9-TET1CD. Complete demethylation was observed at the m2 site in the cells sorted by GFP (Figure 6b). Further analyzation of the cells sorted by GFP for the methylation of a peripheral region showed complete demethylation at the m1 site located 200 bp apart from the target region (Figure 6c). In contrast, the slight demethylation of the m3 and m4 sites located 1 kb or more apart from the target site merely occurred (Figure 6c), and it was suggested that the effect of the demethylation was not greater than that of a site located 1 kb or more apart. In order to test the possibility of targeting of plural of sites, the gRNAs of m1 to m4 were introduced together with System 3 (H19DMR1-4). As a result, roughly complete demethylation was observed in all of the four sites (m1 to m4) in the cells sorted by GFP (Figure 6c). This showed that plural sites can be demethylated by using plural gRNAs.

Example 2. Methylation of Target Using Dnmt3b

[0056] The m2 site of H19 was methylated using System 3 (linker 22aa) in order to introduce methylation into a target. Experiments were conducted using (1) Dnmt3b, (2) Dnmt3bNLS, and (3) Dnmt3bNLS_N662R instead of TET1CD (Figure 7). (1) is a De novo methylase Dnmt3b, (2) is obtained by adding NLS (nuclear localization signal) to the C terminus of the Dnmt3b of (1), and (3) is obtained by changing the 662nd amino acid of (2) from asparagine (N) to arginine (R). This

amino acid substitution has been reported to improve methylation activity (Shen L et al. below). The plasmids used are as follows.

(1) Dnmt3b: pCAG-scFvGCN4sfGFPDnmt3bF (SEQ ID NO: 41)
(2) Dnmt3bNLS: pCAG-scFvGCN4sfGFPDnmt3bFNLS (SEQ ID NO: 42)
(3) Dnmt3bNLS_N662R: pCAG-scFvGCN4sfGFPDnmt3bS1 (SEQ ID NO: 43)

[0057] Only cells into which genes were introduced and which emitted fluorescence were isolated based on fluorescence of GFP by a cell sorter 2 days after introduction of these systems of (1) to (3) into ES cells, and the methylation of the m2 of H19 was examined in a manner similar to that in the demethylation. The methylation was calculated as a methylation (%) standardized by a control, as shown in Numerical Formula 2. As a result, the methylations of the targets were (1) 54%, (2) 74%, and (3) 84%, revealing that methylation efficiency in the case of adding NLS was higher than that in the case of only Dnmt3b, and methylation efficiency in the case of the amino acid substitution of N662R was further higher (Figure 8).

Numerical Formula 2

$$\text{Methylation (\%) standardized by control} = (\text{methylation of sample} - \text{methylation of control})/\text{methylation of control} \times 100$$

References

[0058] Shen L, Gao G, Zhang Y, Zhang H, Ye Z, Huang S, Huang J, Kang J. A single amino acid substitution confers enhanced methylation activity of mammalian Dnmt3b on chromatin DNA. Nucleic Acids Res. 38:6054-6064, 2010. doi: 10.1093/nar/gkq456.

SEQ ID NO: 1: GCN4
SEQ ID NO: 2: linker 5
SEQ ID NO: 3: linker 22
SEQ ID NO: 4: linker 43
SEQ ID NO: 5: 2A peptide
SEQ ID NO: 6: pCAG-dCas9TET1CD
SEQ ID NO: 7: pCAG-dCas9-10xGCN4_v4
SEQ ID NO: 8: pCAG-scFvGCN4sfGFPTET1CD
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SEQ ID NO: 10: pCAG-dCas9-3.5xSuper
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SEQ ID NO: 13: Gfap_2
SEQ ID NO: 14: Gfap_3
SEQ ID NO: 15: H19DMR_1
SEQ ID NO: 16: H19DMR_2
SEQ ID NO: 17: H19DMR_3
SEQ ID NO: 18: H19DMR_4
SEQ ID NO: 19: UR_1
SEQ ID NO: 20: UR_2
SEQ ID NO: 21: UR_3
SEQ ID NO: 22: GfapSTAT3-B3
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SEQ ID NO: 36: H19DMR-B5
SEQ ID NO: 37: H19DMR-B6
SEQ ID NO: 38: off target 1
SEQ ID NO: 39: off target 2
SEQ ID NO: 40: off target 3
SEQ ID NO: 41: pCAG-scFvGCN4sfGFPDnmt3bF
SEQ ID NO: 42: pCAG-scFvGCN4sfGFPDnmt3bFNLS
SEQ ID NO: 43: pCAG-scFvGCN4sfGFPDnmt3bS1
SEQ ID NO: 44: tag peptide GVKESLV
SEQ ID NO: 45: GS linker
SEQ ID NO: 46: GS linker
SEQ ID NO: 47: GS linker

Industrial Applicability

[0059] The methylation of a particular gene can be controlled according to the present invention. As a result, model cells and animals with diseases (epigenome diseases) occurring due to DNA methylation abnormality, such as cancers and imprinting diseases, can be produced. In addition, virus vectors and other delivery systems can be used for treatment of the diseases. In production of iPS cells, the iPS cells can be effectively produced by demethylating and activating a pluripotent gene such as Oct-4 according to the present invention.

SEQUENCE LISTING

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<120> A kit and a method for editing DNA methylation

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<150> JP2015-229896
<151> 2015-11-25

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Claims

1. A DNA methylation editing kit comprising:

   (1) a fusion protein of inactivated CRISPR-associated endonuclease Cas9 (dCas9) having no nuclease activity and a tag peptide array in which a plurality of tag peptides are linked by linkers, or an RNA or DNA coding therefor;
   (2) a fusion protein(s) of a tag peptide-binding portion and a methylase or demethylase, or an RNA(s) or DNA(s) coding therefor; and
   (3) a guide RNA(s) (gRNA(s)) comprising a sequence complementary to a DNA sequence within 1 kb of a desired site of methylation or demethylation, or a DNA(s) expressing the gRNA(s).

2. The DNA methylation editing kit according to claim 1, wherein the demethylase is a catalytic domain (TET1CD) of ten-eleven translocation 1.

3. The DNA methylation editing kit according to claim 1, wherein the methylase is DNA methyltransferase 3 beta (DNMT3B).

4. The DNA methylation editing kit according to any one of claims 1 to 3, wherein the tag peptides are peptide epitopes, and the tag peptide-binding portion is an anti-peptide-epitope antibody.

5. The DNA methylation editing kit according to claim 4, wherein the peptide epitopes are general control non-derepressible 4 (GCN4) peptide epitopes, and the anti-peptide-epitope antibody is an anti-GCN4 peptide epitope antibody.

6. The DNA methylation editing kit according to claim 4, wherein the peptide epitopes are His tags or EE tags, and the anti-peptide-epitope antibody is an anti-His tag antibody or an anti-EE tag antibody.

7. The DNA methylation editing kit according to any one of claims 4 to 6, wherein the antibody is a single-chain antibody (scFv).

8. The DNA methylation editing kit according to any one of claims 1 to 3,
wherein the tag peptides are a small fragment of a split protein, and the tag peptide-binding portion is a large fragment of the split protein.

9. The DNA methylation editing kit according to claim 8, wherein the split protein is GFP.

10. The DNA methylation editing kit according to any one of claims 1 to 3, wherein the tag peptides are GVKESLV (SEQ ID NO: 44), and the tag peptide-binding portion is PDZ protein.

11. The DNA methylation editing kit according to any one of claims 1 to 10, wherein the linkers consist of 5 to 100 amino acids.

12. The DNA methylation editing kit according to any one of claims 1 to 11, wherein the linkers consist of 5 to 50 amino acids.

13. The DNA methylation editing kit according to any one of claims 1 to 12, wherein the linkers consist of 10 to 50 amino acids.

14. The DNA methylation editing kit according to any one of claims 1 to 13, wherein the fusion proteins of the (1) and/or (2) further comprise a selection marker.

15. The DNA methylation editing kit according to any one of claims 1 to 14, which contains plural gRNAs.

16. The DNA methylation editing kit according to any one of claims 1 to 15, wherein all the DNAs of the (1) to (3) are contained in a vector.

17. A DNA methylation editing method comprising transfecting a cell with the following (1) to (3):

    (1) a fusion protein of inactivated CRISPR-associated endonuclease Cas9 (dCas9) having no nuclease activity

and a tag peptide array in which a plurality of tag peptides are linked by linkers, or an RNA or DNA coding therefor;
(2) a fusion protein(s) of a tag peptide-binding portion and a methylase or demethylase, or an RNA(s) or DNA(s) coding therefor; and
(3) a guide RNA(s) (gRNA(s)) comprising a sequence complementary to a DNA(s) sequence within 1 kb of a desired site of methylation or demethylation, or a DNA expressing the gRNA(s).

18. The DNA methylation editing method according to claim 17, wherein the fusion proteins of the (1) and/or (2) further comprise a selection marker.

19. The DNA methylation editing method according to claim 18, further comprising selecting and collecting a cell expressing the selection marker.

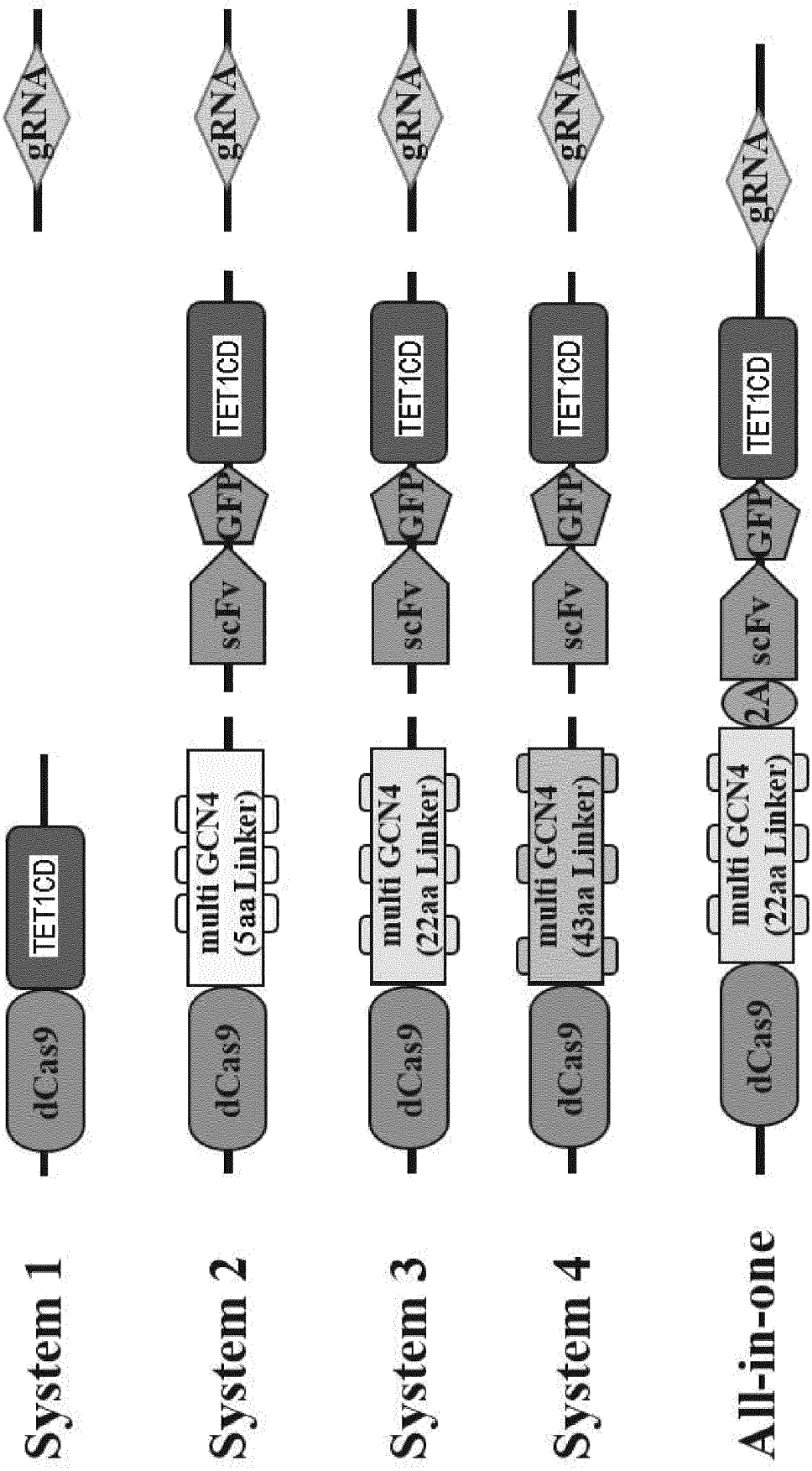
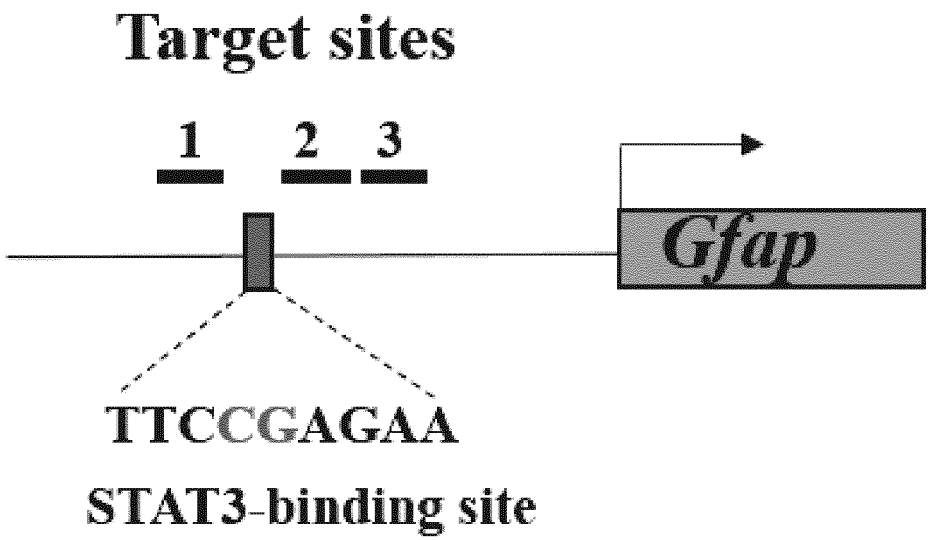

System 1
dCas9
TET1CD
gRNA
System 2
dCas9
multi GCN4 (5aa Linker)
scFv
GFP
TET1CD
gRNA
System 3
dCas9
multi GCN4 (22aa Linker)
scFv
GFP
TET1CD
gRNA
System 4
dCas9
multi GCN4 (43aa Linker)
scFv
GFP
TET1CD
gRNA
All-in-one
dCas9
multi GCN4 (22aa Linker)
2A
scFv
GFP
TET1CD
gRNA

FIG. 1

(a)


Target sites
1
2
3
Gfap
TTCCGAGAA
STAT3-binding site

(b)

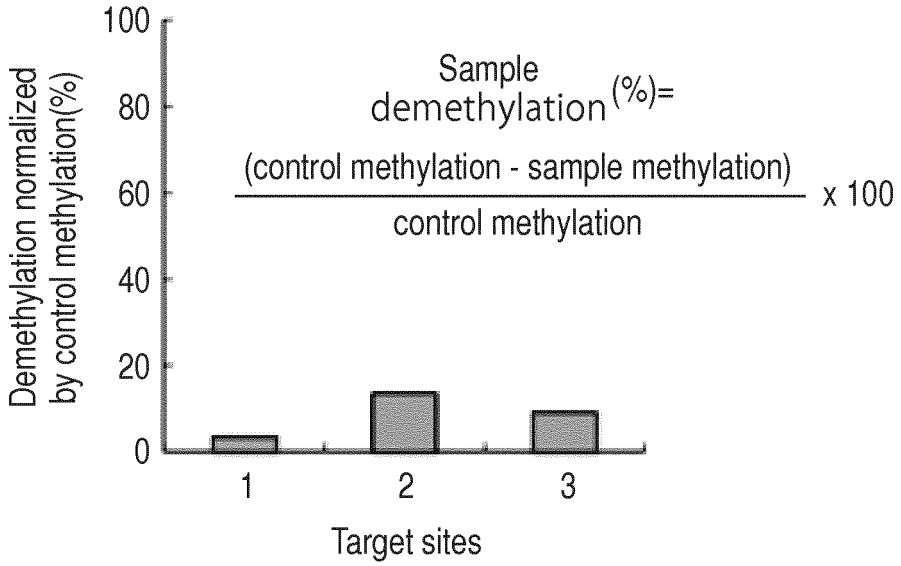

Demethylation normalized by control methylation(%)
100
80
60
40
20
0
Sample demethylation (%)=
(control methylation - sample methylation) / control methylation x 100
1
2
3
Target sites

FIG.2

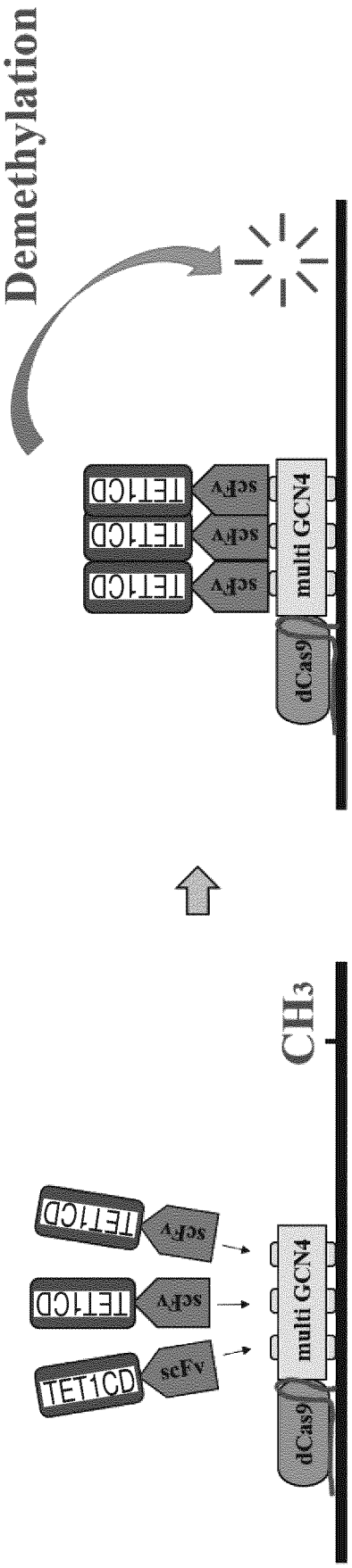


FIG.3A

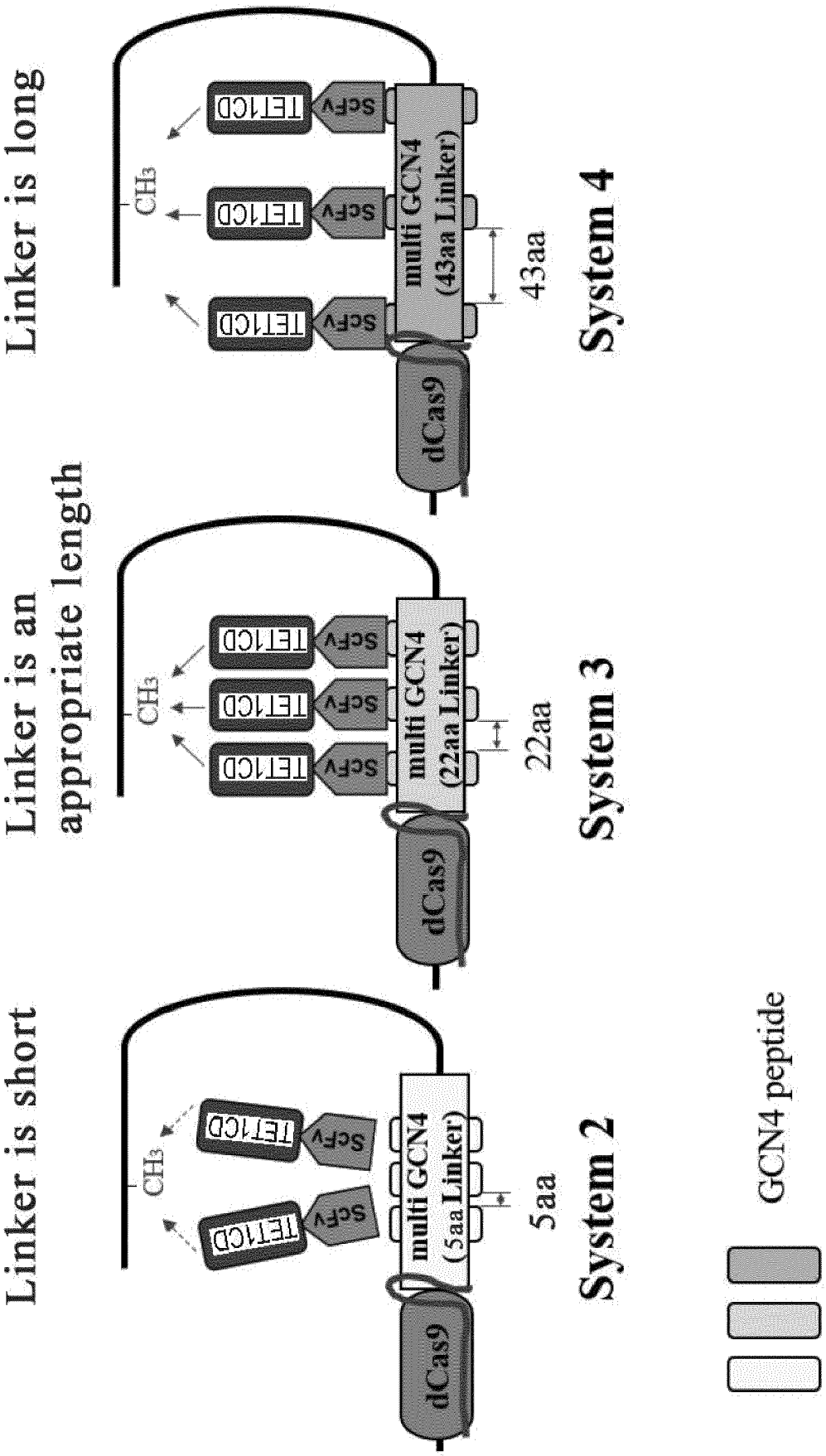

Linker is short
Linker is an appropriate length
Linker is long
System 2
System 3
System 4
dCas9
multi GCN4 (5aa Linker)
multi GCN4 (22aa Linker)
multi GCN4 (43aa Linker)
5aa
22aa
43aa
ScFv
TET1CD
CH3
GCN4 peptide

FIG.3B

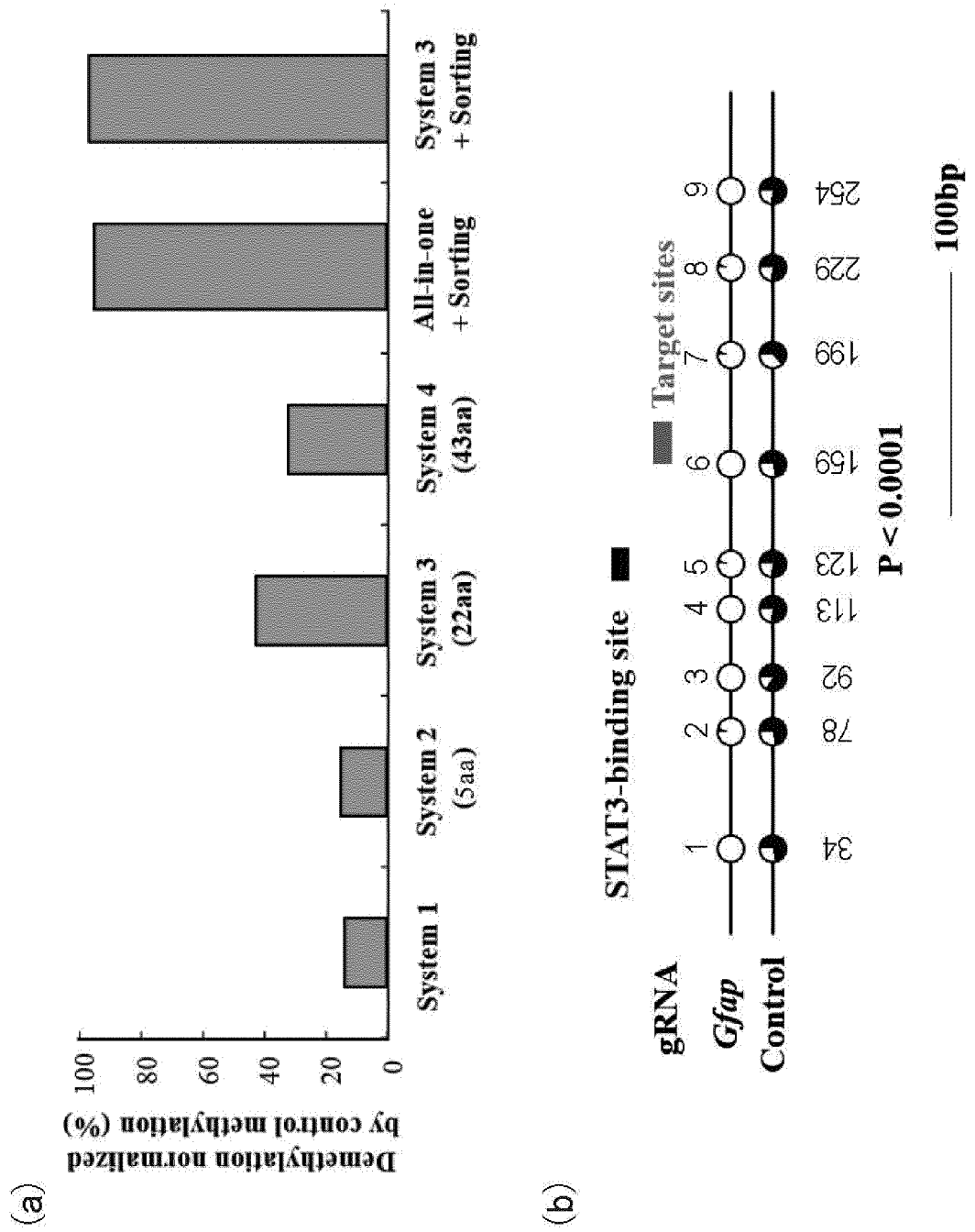

(a)
Demethylation normalized by control methylation (%)
100
80
60
40
20
0
System 1
System 2 (5aa)
System 3 (22aa)
System 4 (43aa)
All-in-one + Sorting
System 3 + Sorting
(b)
STAT3-binding site
Target sites
gRNA
Gfap
Control
1
2
3
4
5
6
7
8
9
34
78
92
113
123
159
199
229
254
P < 0.0001
100bp

FIG.4

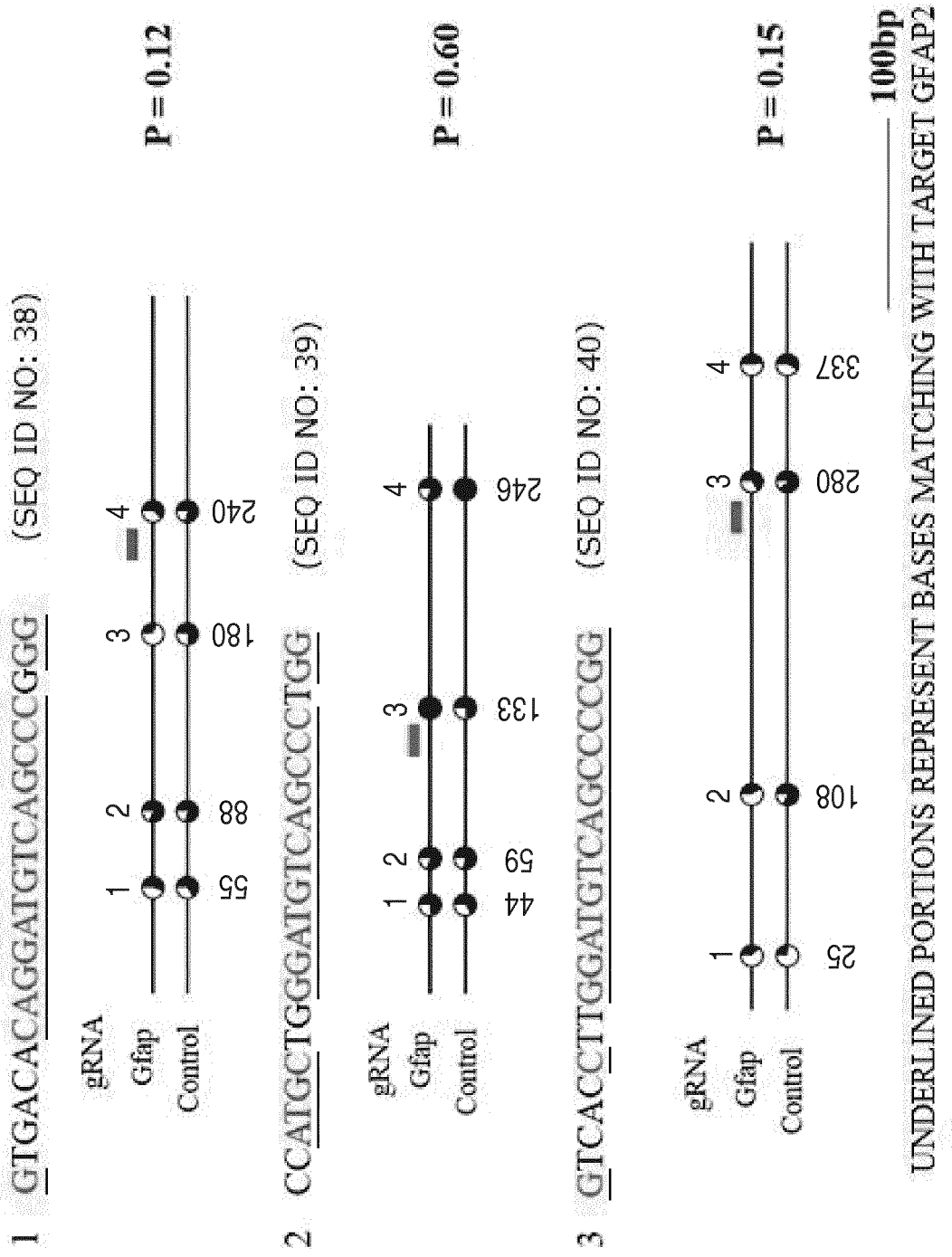

1 GTGACACAGGATGTCAGCCCGGG (SEQ ID NO: 38)
gRNA
Gfap
Control
1 2 3 4
55 88 180 240
P = 0.12
2 CCATGCTGGGATGTCAGCCCTGG (SEQ ID NO: 39)
gRNA
Gfap
Control
1 2 3 4
44 59 133 246
P = 0.60
3 GTCACCTTGGATGTCAGCCCCGG (SEQ ID NO: 40)
gRNA
Gfap
Control
1 2 3 4
25 108 280 337
P = 0.15
100bp
UNDERLINED PORTIONS REPRESENT BASES MATCHING WITH TARGET GFAP2
Target homology

FIG.5

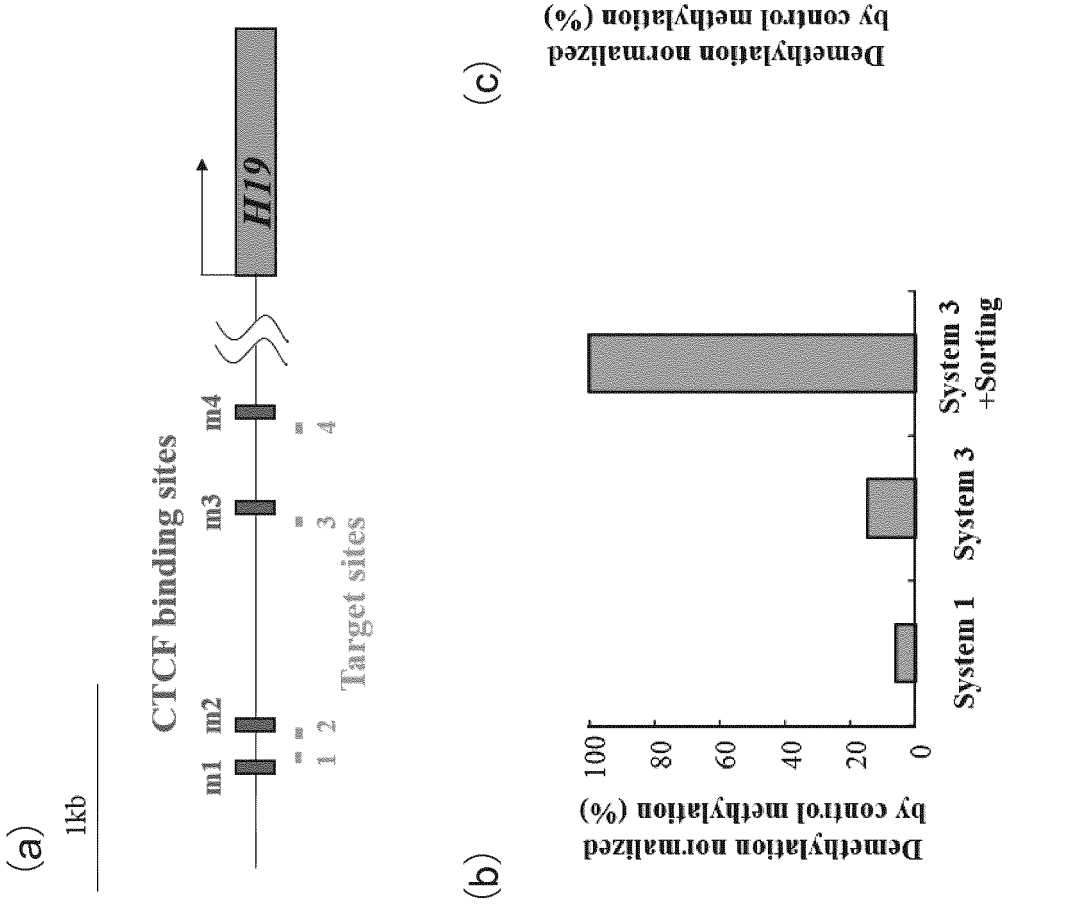

(a)
1kb
CTCF binding sites
m1
m2
m3
m4
1
2
3
4
Target sites
H19
(b)
Demethylation normalized by control methylation (%)
0
20
40
60
80
100
System 1
System 3
System 3 +Sorting
(c)
Demethylation normalized by control methylation (%)
0
20
40
60
80
100
m1
m2
m3
m4
Target 2
Target 1-4

FIG.6

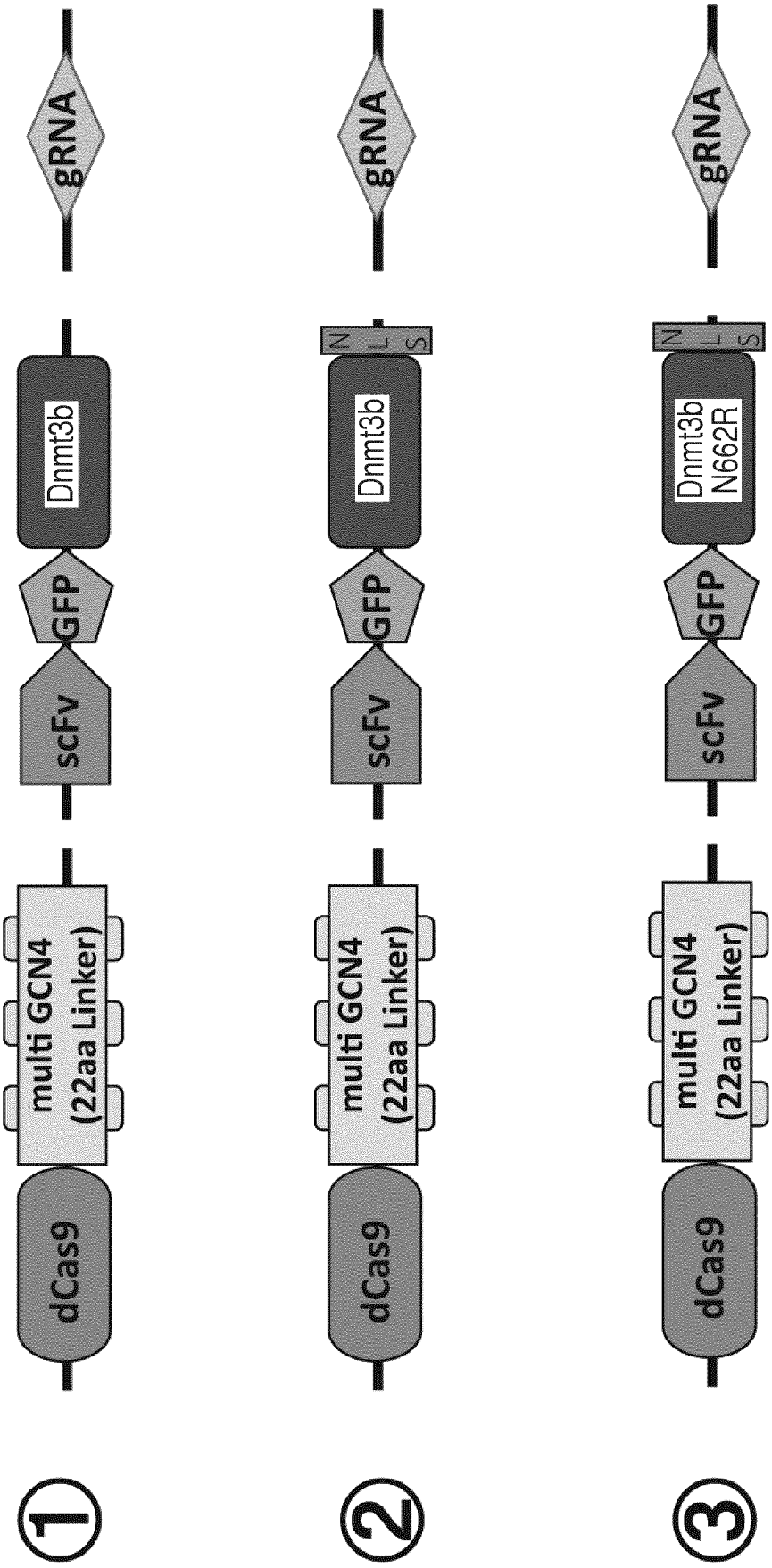

gRNA
Dnmt3b
GFP
scFv
multi GCN4
(22aa Linker)
dCas9
1
gRNA
NLS
Dnmt3b
GFP
scFv
multi GCN4
(22aa Linker)
dCas9
2
gRNA
NLS
Dnmt3b
N662R
GFP
scFv
multi GCN4
(22aa Linker)
dCas9
3

FIG.7

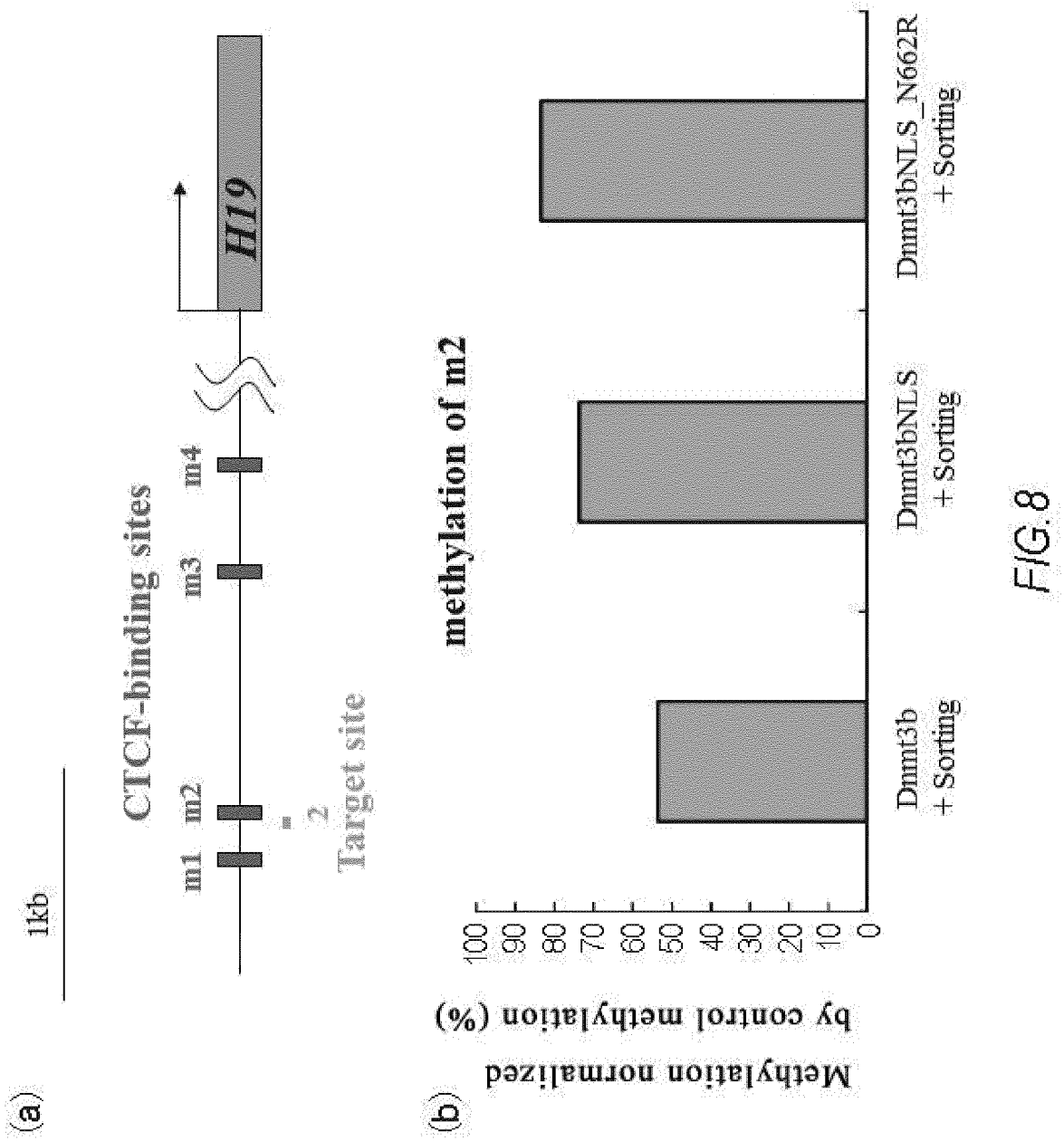

(a)
1kb
CTCF-binding sites
m1
m2
m3
m4
H19
2
Target site
(b)
Methylation normalized by control methylation (%)
methylation of m2
100
90
80
70
60
50
40
30
20
10
0
Dnmt3b + Sorting
Dnmt3bNLS + Sorting
Dnmt3bNLS_N662R + Sorting

FIG.8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2016/084958

A. CLASSIFICATION OF SUBJECT MATTER
C12N15/09(2006.01)i, *C07K16/18*(2006.01)i, *C12N1/15*(2006.01)i, *C12N1/19*(2006.01)i, *C12N1/21*(2006.01)i, *C12N5/10*(2006.01)i, *C12N9/10*(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N15/09, C07K16/18, C12N1/15, C12N1/19, C12N1/21, C12N5/10, C12N9/10

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2017
Kokai Jitsuyo Shinan Koho 1971-2017 Toroku Jitsuyo Shinan Koho 1994-2017

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus/JMEDPlus/JST7580(JDreamIII), CAplus/MEDLINE/BIOSIS/WPIDS(STN), DWPI(Thomson Innovation)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TANENBAUM E. M. et al., A Protein-Tagging System for Signal Amplification in Gene Expression and Fluorescence Imaging, Cell, 2014, Vol.159, pp.635-646	1-19
Y	MAEDER M. L. et al., Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins, Nature Biotechnology, 2013, Vol.31, No.12, pp.1137-1142	1-19
Y	MALI P. et al., Cas9 as a versatile tool for engineering biology, Nature Methods, Vol.10, No.10, 2013, pp.957-963	1-19

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 2017 (16.01.17)	31 January 2017 (31.01.17)
Name and mailing address of the ISA/ Japan Patent Office 3-4-3,Kasumigaseki,Chiyoda-ku, Tokyo 100-8915,Japan	Authorized officer Telephone No.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2016/084958

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Taiga YAMAZAKI et al., "Genome Henshu Gijutsu o Oyo shita Pericentromere eno Jin'iteki·Hairetsu Tokuteki DNA Methyl-ka Yudo", BMB2015 Koen Yoshishu, 06 November 2015 (06.11.2015), vol.2015th, #1P0832	1-19
Y	WO 2014/152432 A2 (THE GENERAL HOSPITAL CORP.), 25 September 2014 (25.09.2014), claims 7 to 9, 24; page 22, lines 17 to 18 & US 2014/0295556 A1 & US 2014/0295557 A1 & EP 2970986 A2 & KR 10-2015-0131250 A & CN 105408483 A	1-19
Y	SHEN L. et al., A single amino acid substitution confers enhanced methylation activity of mammalian Dnmt3b on chromatin DNA, Nucleic Acids Research, 2010, Vol.38, No.18, pp.6054-6064	3-19
Y	CABANTOUS S. et al., A new protein-protein interaction sensor based on tripartite split-GFP association, Scientific Reports, 2013, Vol.3, pp.2854/1-2854/9	8-9
Y	SKELTON N. J. et al., Origins of PDZ domain ligand specificity, The Journal of Biological Chemistry, 2003, Vol.278, No.9, pp.7645-7654	10
P,X/P,Y	MORITA S. et al., Targeted DNA demethylation in vivo using dCas9-peptide repeat and scFv-TET1 catalytic domain fusions, Nature Biotechnology, 2016.10, Vol.34, No.10, pp.1060-1065, Epub: 29 AUG 2016	1-2,4-7, 11-19/3,8-10

Form PCT/ISA/210 (continuation of second sheet) (January 2015)

REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description

- **MAEDER ML et al.** *Nat Biotechnol,* 2013, vol. 31, 1137-1142 **[0005]**
- JIKKEN IGAKU. YODOSHA CO., LTD, July 2014, 1690-1714 **[0005]**
- **TANENBAUM ME et al.** *Cell,* 2014, vol. 159, 635-646 **[0005]**
- *Protein Engineering, Design & Selection,* 2011, vol. 24 (5), 419-428 **[0020]**
- *Current Opinion in Chemical Biology,* 2011, vol. 15, 789-797 **[0021]**
- *Proc. Natl. Acad. Sci. USA,* 2008, vol. 105 (18), 6578-6583 **[0023]**
- **SHEN L ; GAO G ; ZHANG Y ; ZHANG H ; YE Z ; HUANG S ; HUANG J ; KANG J.** A single amino acid substitution confers enhanced methylation activity of mammalian Dnmt3b on chromatin DNA. *Nucleic Acids Res.,* 2010, vol. 38, 6054-6064 **[0058]**